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Bioorganic & Medicinal Chemistry 13 (2005) 4821–4834
Bioorganic & Medicinal Chemistry

Insight into 2-phenylpyrazolo[1,5-*a*]pyrimidin-3-yl acetamides as peripheral benzodiazepine receptor ligands: Synthesis, biological evaluation and 3D-QSAR investigation

Silvia Selleri,[a] Paola Gratteri,[a,*] Camilla Costagli,[a] Claudia Bonaccini,[a] Annarella Costanzo,[a] Fabrizio Melani,[a] Gabriella Guerrini,[a] Giovanna Ciciani,[a] Barbara Costa,[b] Francesca Spinetti,[b] Claudia Martini[b] and Fabrizio Bruni[a]

[a]*Dipartimento di Scienze Farmaceutiche, Università di Firenze, Via U. Schiff, 6, 50019 Sesto F.no Firenze, Italy*
[b]*Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, Università di Pisa, Via Bonanno 6, 56126 Pisa, Italy*

Received 18 October 2004; revised 4 May 2005; accepted 10 May 2005
Available online 21 June 2005

Abstract—The present paper reports the synthesis and binding studies of new 2-phenylpyrazolo[1,5-*a*]pyrimidin-3-yl acetamides as selective Peripheral Benzodiazepine Receptor (PBR) ligands. The variability of substituents at the 3-position was investigated and a 3D-QSAR model was proposed to evaluate the effect of different substitutions on the acetamide moiety. In addition, a subset of the novel compounds showing high affinity for PBR was tested for their ability to modulate the steroid biosynthesis in C6 glioma cells.

1. Introduction

The peripheral benzodiazepine receptor (PBR) is a protein of 18 kDa, which can form a trimeric complex with the voltage dependent anion channel VDAC (32 kDa) and adenine nucleotide carrier ANC (30 kDa).[1] Two additional proteins (10 kDa and PRAX-1) are believed to be involved in modulating PBR functions.[2] The 18 kDa subunit was identified, cloned and expressed, initially in rat and subsequently in mouse, bovine and human species. Among these, the 18 kDa protein, consisting of 169 amino acids, shows about 80% amino acid conservation.[3]

The PBR was found in the majority of peripheral tissues, in blood cells and also in astrocytes, even if the density of the PBR is the highest in endocrine tissues such as adrenal gland, testis and ovary.[4] This receptor is mainly located in the outer membrane of mitochondria, but there is evidence of its presence elsewhere in the cell.[5]

The PBR is implied in numerous biological processes, even if the precise function of this receptor remains an enigma; however, considering its ubiquitous distribution and high conservation in the evolutionary scale, it is suggested that PBR may serve as a target to control various mitochondrial and cell functions and probably to protect against toxic events.[6,7] In addition, binding studies indicated that PBR density can be modulated under a variety of physiological and pathological conditions.[8] However to date, among the many functions attributed to the PBR, its involvement in biosynthesis of steroids is the most investigated.[9–13] The PBR is involved in the transport of cholesterol into mitochondria, which represents the rate-limiting step of the whole steroid biosynthesis process. Steroidogenesis begins with the conversion of cholesterol to pregnenolone by the cytochrome P-450 side chain cleavage ($P450_{scc}$), which is located at the inner mitochondrial membrane. Most studies report that PBR ligands stimulate steroids production.[12] Nonetheless, a polypeptide of seven amino acids was identified as a new PBR ligand able to antagonize the steroid biosynthesis and it could represent a useful lead for synthesizing new antagonist PBR ligands, the target of the current research.[14]

Nowadays, a wide variety of endogenous molecules with affinity for the PBR have been identified, such as DBI

Keywords: Peripheral benzodiazepine receptor; Pyrazolo[1,5-*a*]pyrimidines; 3D QSAR; Steroidogenesis assay.
* Corresponding author. Tel.: +39 55 457302; fax: +39 55 4573671; e-mail: paola.gratteri@unifi.it

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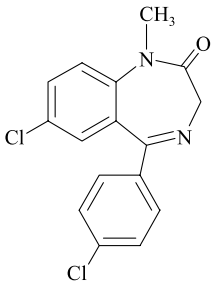
doi:10.1016/j.bmc.2005.05.015

(Diazepam Binding Inhibitor), which is a 11 kDa polypeptide of 86 amino acids, and porphyrins (protoporphyrin IX, mesoporphyrin IX, deuteroporphyrin IX and hemin).[2]

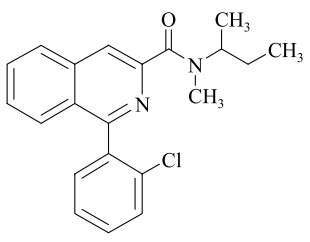
In addition, the PBR exhibits nanomolar affinity to benzodiazepine Ro5-4864 (4′-chlorodiazepam), but very low affinity to other benzodiazepines; moreover, isoquinolines (PK11195), imidazopyrimidines, indole derivatives, pirrolobenzoxazepines and phenoxyphenyl acetamides have been recognized as exogenous PBR ligands. Particularly, it is reported that isoquinolines, such as PK11195, interact specifically with the 18 kDa protein component, whereas PBR-specific benzodiazepines seem to require the interaction of all three protein components (18 kDa, VDAC, ANC) for binding. Indeed, the well-known ligands PK11195 and Ro5-4864 (Chart 1) compete with each other in binding experiments, suggesting overlapping but not identical binding sites.[15–17]

In addition to site-direct mutagenesis studies,[15] useful information on the PBR are supplied by several computational analyses.[18–21] These try to rationalize the variation in the binding affinity of the large variety of ligands and to identify the crucial amino acids involved in the binding of synthetic and endogenous ligands. All existing QSAR studies in the literature agree with the need for two hydrophobic interaction areas and a H-bond donating site in the PBR receptor as essential requirements for the ligand binding. Among the proposed models only a few are derived starting from series of structurally different compounds;[18] most of them are obtained on congeneric series of molecules.[19–21] However, with some limitations (which mainly refer to the local application on the series from which they are derived), these models represent a reliable tool in both the description of the molecules used to build the model and in the prediction of the biological activities of analogues not yet synthesized.

In a previous paper,[22] we described the results of our research on PBR ligands, which led to discovery of a new class of 2-arylpyrazolo[1,5-*a*]pyrimidin-3-yl acetamides, azaisosters of Alpidem, endowed with high affinity and PBR selectivity (Chart 2).

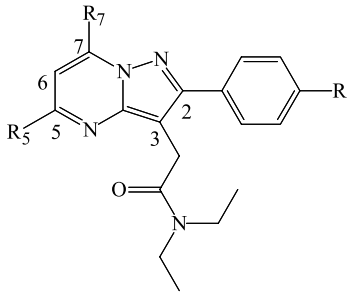


Ro 5-4864

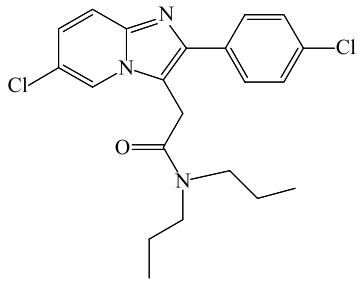


PK 11195

Chart 1.



N,N-dietil-2-arylpyrazolo[1,5-*a*]pyrimidin-3-yl acetamides



Alpidem

Chart 2.

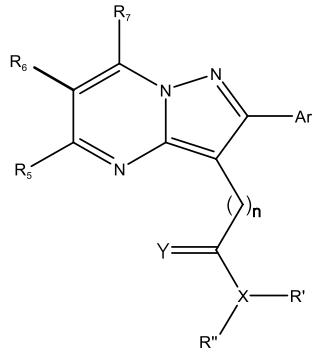
Mainly, the 5 and 7 positions were investigated, and from preliminary SAR studies it emerged that substitutions on the pyrimidine moiety seem to be the key factor promoting the PBR versus CBR (Central Benzodiazepine Receptor) selectivity. Recently, as an extension of our studies on pyrazolo[1,5-*a*]pyrimidines as PBR ligands, our attention has been focused on the 3 position to clarify the role of the amide moiety and the main structural features concerning the number and the length of the alkyl substituents on the amide nitrogen. Indeed, it has been reported[18–21] that hydrogen bond interaction of a polar group with the receptor protein seems to be of paramount importance for the binding to the PBR. In this paper, we report the synthesis of new 2-phenyl-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl acetamides and their biological evaluation in order to investigate PBR affinity and PBR versus CBR selectivity. In addition, a 3D-QSAR model using the GRID/Golpe[23] procedure is proposed to evaluate the effect of the substitution on acetamide chain. Previously published compounds are also included (Table 1).

A subset of the synthesized compounds showing high affinity and selectivity for PBR was also evaluated for their ability to modulate the steroid biosynthesis in C6 glioma cells, since this test is currently the only reliable method to prove the affinity and/or intrinsic activity of PBR ligands.

2. Chemistry

The synthesis of compounds **3b** and **4a–q** (Scheme 1) started from the common intermediate, benzoylacetonitrile, which reacted in basic medium ($LiOH{\cdot}H_2O$) with iodoacetic acid or ethyl iodoacetate to give the

Table 1. Structures and PBR/CBR affinity values of all compounds reported in this paper



Compound	*n*	X	Y	Ar	R_5	R_6	R_7	R′	R″	PBR K_i (nM)[a]	CBR K_i (nM)[b]
3f[c]	1	N	O	Ph	Me	H	H	Et	Et	40	92
3g[c]	1	N	O	Ph	H	H	Me	Et	Et	155	518
3i[c]	1	N	O	Ph	Me	H	Me	Et	Et	29	251
3j[c]	*1*	*N*	*O*	*4Cl–Ph*	*Me*	*H*	*Me*	*Et*	*Et*	*2.4*	*>10.00*
3k[c]	*1*	*N*	*O*	*4F–Ph*	*Me*	*H*	*Me*	*Et*	*Et*	*9.2*	*>10.00*
3l[c]	*1*	*N*	*O*	*4Me–Ph*	*Me*	*H*	*Me*	*Et*	*Et*	*0.8*	*>10.00*
3m[c]	*1*	*N*	*O*	*4MeO–Ph*	*Me*	*H*	*Me*	*Et*	*Et*	*4.7*	*>10.00*
3n[c]	1	N	O	4F–Ph	Me	H	H	Et	Et	25	85
3o[c]	*1*	*N*	*O*	*4Cl–Ph*	*H*	*H*	*Me*	*Et*	*Et*	*8.1*	*207*
3p[c]	1	N	O	4Me–Ph	Me	H	CF_3	Et	Et	0.9	5800
3q[c]	1	N	O	4Me–Ph	CF_3	H	Me	Et	Et	1	6900
3r[c]	*1*	*N*	*O*	*4MeO–Ph*	*CF_3*	*H*	*CF_3*	*Et*	*Et*	*16*	*6260*
3s[c]	*1*	*N*	*O*	*4Cl–Ph*	*Me*	*H*	*Ph*	*Et*	*Et*	*2.4*	*>10.00*
3t[c]	*1*	*N*	*O*	*4Cl–Ph*		*H*	*Me*	*Et*	*Et*	*3.4*	*>10.00*
3u[c]	1	N	O	4Cl–Ph	Me	Me	Me	Et	Et	6.1	>10.00
3v[c]	*1*	*N*	*O*	*4Cl–Ph*	*Me*	*COOEt*	*Me*	*Et*	*Et*	*8.4*	*>10.00*
3x[c]	*1*	*N*	*O*	*4Me–Ph*	*H*	*Ph*	*H*	*Et*	*Et*	*57*	*>10.00*
3y[c]	1	N	O	Ph	Ph	H	H	Et	Et	225	>10.00
4[d]	*1*	*N*	*O*	*4Me-Ph*	*Me*	*H*	*H*	*Me*	*Me*	*1192*	*255*
3b	*1*	*O*	*O*	*Ph*	*Me*	*H*	*Me*	*Et*		*1610*	*>10.00*
4a	*1*	*N*	*O*	*Ph*	*Me*	*H*	*Me*	*Me*	*Me*	*710*	*>10.00*
4b	*1*	*N*	*O*	*Ph*	*Me*	*H*	*Me*	*n-Pr*	*n-Pr*	*0.8*	*>10.00*
4c	*1*	*N*	*O*	*Ph*	*Me*	*Me*	*Me*	*i-Pr*	*i-Pr*	*89*	*10.00*
4d	*1*	*N*	*O*	*Ph*	*Me*	*H*	*Me*	*n-But*	*n-But*	*4.5*	*10.00*
4e	*1*	*N*	*O*	*Ph*	*Me*	*H*	*Me*	*n-pentyl*	*n-pentyl*	*8*	*10.00*
4f	*1*	*N*	*O*	*Ph*	*Me*	*H*	*Me*	*n-hexyl*	*n-hexyl*	*23*	*10.00*
4g	*1*	*N*	*O*	*Ph*	*Me*	*H*	*Me*	*n-octyl*	*n-octyl*	*1124*	*10.00*
4h	1	N	O	Ph	Me	H	Me	Bz	Bz	40	>10.00
4i	*1*	*N*	*O*	*Ph*	*Me*	*H*	*Me*	*Et*	*i-Pr*	*28*	*10.00*
4j	*1*	*N*	*O*	*Ph*	*Me*	*H*	*Me*	*Et*	*Ph*	*0.8*	*10.00*
4k	*1*	*N*	*O*	*Ph*	*Me*	*H*	*Me*	*Et*	*Bz*	*7.3*	*10.00*
4l	*1*	*N*	*O*	*Ph*	*Me*	*H*	*Me*	*Me*	*(R)-$CHCH_3$Ph*	*22*	*10.00*
4m	*1*	*N*	*O*	*Ph*	*Me*	*H*	*Me*	*Me*	*(S)-$CHCH_3$Ph*	*156*	*10.00*
4n	1	N	O	Ph	Me	H	Me		–$(CH_2)_4$–	1154	>10.00
4o	1	N	O	Ph	Me	H	Me		–$(CH_2)_5$–	304	>10.00
4p	*1*	*N*	*O*	*Ph*	*Me*	*H*	*Me*		*–$CH_2CH_2N(Me)CH_2CH_2$–*	>10,000	*10.00*
4q	*1*	*N*	*O*	*Ph*	*Me*	*H*	*Me*	*n-Pr*	*H*	*376*	*10.00*
5	*1*	*N*	*S*	*Ph*	*Me*	*H*	*Me*	*Et*	*Et*	*44*	*10.00*
8	0	N	O	Ph	Me	H	Me	Et	Et	270[e]	>10.00

Italic entries represent compounds in the training set.

[a] K_i values represent means ± SEM derived from three independent experiments, conducted in triplicate using 0.6 nM [^{3}H]PK11195 as the radioligand.

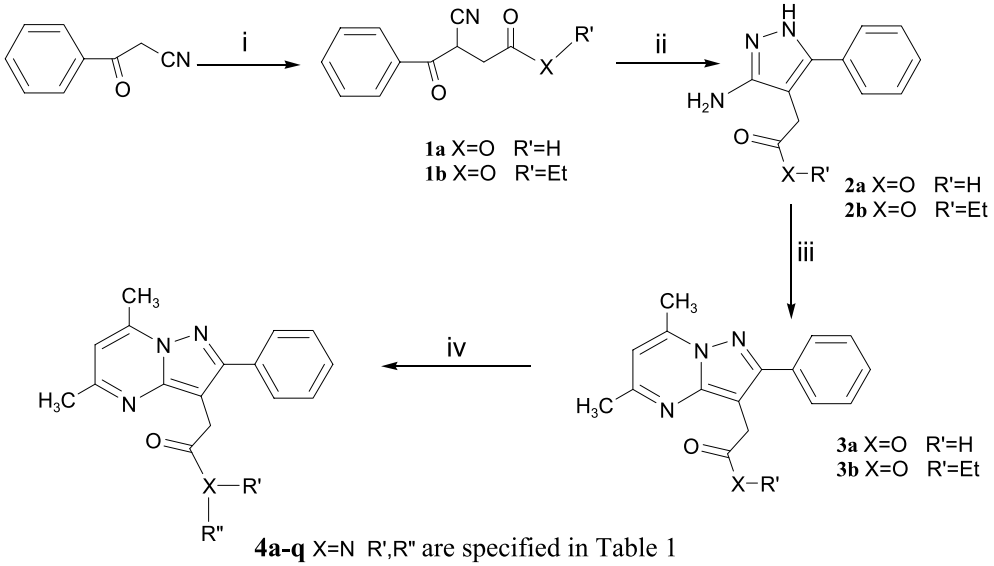
[b] Percent inhibition values of specific [^{3}H]Ro15-1788 binding at 0.2 nM concentration are means ± SEM of five separate experiments, each done in triplicate.

[c] See Ref. 22.

[d] See Ref. 35.

[e] Affinity values were calculated using 1 nM [^{3}H]Ro 5-4864 as the radioligand.

corresponding acid (**1a**) and ester (**1b**), respectively. The achievement of compounds **1a** and **b** was performed by exploiting the base-promoted reactivity of methylene group of the benzoylacetonitrile, which reacts with elec-



Scheme 1. Reagents and conditions: (i) iodoacetic acid/LiOH·H_2O/EtOH 80% (method A), ethyl iodoacetate/thallium ethylate/tol (method B); (ii) N_2H_4·H_2O/EtOH/AcOH; (iii) 2,4-pentanedione/EtOH; (iv) NEt_3/ClCOOEt/THF/suitable amines reported in Experimental section.

tron-poor carbon atom to give 1,4-dicarbonyl compounds.

Compound **1a** was obtained in good yield (70%) using LiOH·H_2O as base in 80% EtOH (method A), whereas compound **1b** was synthesized employing ethyl iodoacetate in the presence of thallium ethoxide in toluene (method B).[24] A previously published method for the synthesis of the ester derivative was followed,[25] using Hunig's base (DIEA, diisopropylethylamine) and anhydrous magnesium chloride in dichloromethane. Nevertheless, following this procedure the yield of compound **1b** resulted unsatisfactorily; therefore the alternative method B was used.

In the second step of this synthetic route, the intermediates **1a** and **b** reacted in ethanol at reflux with hydrazine hydrate, in the presence of acetic acid, to give the corresponding 3-amino pyrazoles (**2a** and **b**) in good yield and purity.

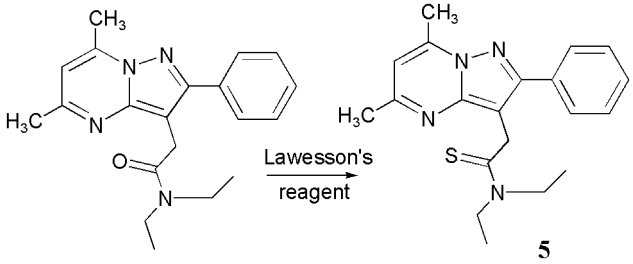
In the third step, the condensation of compounds **2a** and **b** with 2,4-pentanedione was carried out, which led to the closure of the pyrimidine ring, resulting in the intermediate **3a** and the final compound **3b**. Subsequently, the acid **3a** was converted into a mixed anhydride with ethyl chloroformate, and this intermediate reacted with a large number of amines affording the corresponding amides (**4a**–**q**) in good yields and in a short time.

Several attempts were made to convert the acid **3a** into its chlorocarbonyl derivative. However, thionyl chloride and diethylchlorophosphate,[26] which are generally used for preparing acyl chlorides from carboxylic acids, cannot be used in this case due to the generation of strong acid conditions during the reaction, which probably destroy the bicyclic nucleus. Therefore, we considered the reaction of trichloroacetonitrile (TCA) with triphenylphosphine, which generates triarylphosphonium chloride,[27] and the method which uses *N*-halosuccinimides (i.e., NCS and NBS) in combination with triphenylphosphine.[28] These methods represent two valid approaches for converting pyrazolo[1,5-*a*]pyrimidin-3-yl acetic acids to the corresponding amides; nevertheless, the best yields were obtained applying the method via mixed anhydride.

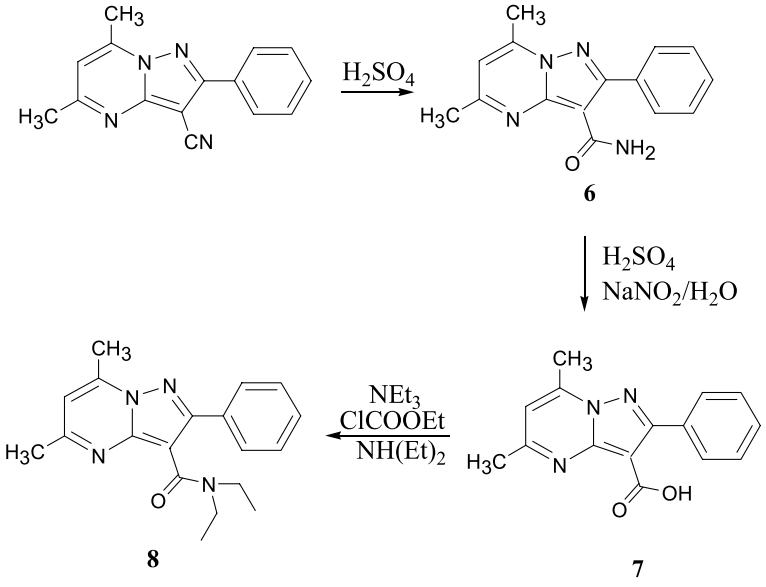
Compound **5** was synthesized from *N*,*N*-diethyl-5,7-dimethyl-2-phenylpyrazolo[1,5-*a*]pyrimidin-3-yl acetamide[22] by reaction with Lawesson's reagent (Scheme 2); compound **8** (Scheme 3) was prepared starting from 5,7-dimethyl-2-phenylpyrazolo[1,5-*a*]pyrimidine-3-carbonitrile[29] by treatment with H_2SO_4 to provide the corresponding amide (**6**), which was further hydrolyzed to the corresponding carboxylic acid. Finally, compound **7** was converted to the *N*,*N*-diethylcarboxyamide (**8**) following the above mentioned procedure for the synthesis of compounds **4a**–**q**.

3. Results and discussion

The ability of compounds **3b**, **4a**–**q**, **5** and **8** to interact with the PBR was investigated by a binding assay using [^{3}H]PK11195 as the radioligand and membranes from



Scheme 2.



Scheme 3.

rat kidney tissues as receptor source; the tested compounds display a broad range of binding affinities as shown in Table 1. The newly synthesized PBR ligands have also been examined for their receptor selectivity by evaluating their ability to displace [^{3}H]Ro15-1788 from Bz/GABA$_A$ receptors and none of the compounds were found to exhibit any significant binding affinity for the CBR. The lack of CBR recognition observed in all the synthesized compounds seems to confirm our previous hypothesis that the substitution on the pyrimidine moiety could be the key in promoting the PBR selectivity.[22] In fact, in designing the new PBR ligands, we started from the lead compound, *N*,*N*-diethyl-2-phenyl-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl acetamide (**3i**),[22] and we decided to maintain the phenyl ring at the 2-position and two methyl groups at the 5- and 7-positions of the pyrazolo[1,5-*a*]pyrimidine nucleus because this di-substitution on pyrimidine ring resulted in favouring the PBR versus CBR selectivity. In this paper, our attention is focused on the 3-position of the bicyclic nucleus to investigate the role of the amide moiety and the requirements concerning the number and the length of the alkyl substituents on the amide nitrogen.

Analyzing the binding results, it emerged that the chain length of alkyl groups on the amide nitrogen is important for binding affinity, as can be observed in compounds **4a** (methyl), **3i** (ethyl),[22] **4b** (*n*-propyl), **4d** (*n*-butyl), **4e** (*n*-pentyl), **4f** (*n*-hexyl) and **4g** (*n*-octyl). Among these compounds, the best result was shown by compound **4b** bearing three carbon atoms in the amide moiety. Branching of the chain was unfavourable, as is shown by compounds **4c** and **4i**. Moreover, the di-substitution of the amide nitrogen is clearly preferred; indeed **4q**, bearing a single *n*-propyl group, shows a low PBR binding affinity. Comparable results have been obtained with the introduction of cyclic amines.[30,31] In fact, the attempts to explore stiffening of alkyl substituents on the amide nitrogen with the synthesis of cyclic amides led to compounds which exhibit low affinity (**4n** and **4o**) or even lack of recognition at the PBR (**4p**). On the other hand, the introduction of phenyl ring on the amide nitrogen positively contributes to increase the binding affinity (**4j**), while the presence of spacers between amide nitrogen and the aromatic ring results in decreased affinity values (**4h**, **4k**, **4l** and **4m**). Interestingly, the insertion of one asymmetric carbon atom in the acetamide moiety determines a very strong difference in binding affinity between the stereoisomers **4l** and **4m** and this evidence suggests that suitably oriented lipophylic substituents play a substantial role in binding affinity. This observation is in good agreement with the only available studies on stereoselectivity of PK11195 published between the end of the 1980s and 1994[32–34] in which the *R*-stereoisomer was observed to be approximately 2.5-fold more potent than its enantiomer. This stereoselectivity was observed in all the tissues tested (brain, heart, kidney and adrenals) and it was found also in vivo, suggesting that the use of R-enantiomer would have advantages over the use of the racemate currently employed.

In the series of pyrazolo[1,5-*a*]pyrimidines, removal of the methylene linker between the pyrazolopyrimidine nucleus and the amide group (compound **8**) as well as the replacement of this latter with the ester function (**3b**), resulted in a notable decrease in binding potency.[31] At first glance, this result seems to confirm the essential role of the 3-acetamide moiety for PBR interaction. Nevertheless, the maintenance of good affinity value exerted by compound **5**, bearing the tioacetamide moiety which is unable to stabilize hydrogen bond interaction, forces revision of the role of the acetamido group at the 3-position. It could be hypothesized that the acetamide moiety might not be essential in stabilizing the H-bond with the receptor protein, but it represents an optimal carrier because its length and orientation allow lipophylic groups to fit with the proper lipophylic

pockets on the receptor protein. Moreover, it should be considered that the N^4 atom may represent an alternative H-bond acceptor group in this series, if the group at the 3-position is unable to form this interaction.

3.1. 3D-QSAR analysis

All the 38 pyrazolopyrimidines reported in Table 1 (19 compounds presented in this paper plus 18 already published,[22] together with compound **4**[35]) fulfill the requirements for the PBR recognition, that is, the ability to interact by a H-bond with the receptor protein and the presence of hydrophobic groups. This series of PBR ligands was investigated according to a Grid/Golpe procedure focusing our attention on evaluating the effects of the substitutions on the acetamide chain.

Probes C3 and N1 were selected for the Grid/Golpe analysis. The DRY probe provides almost the same information as the C3 probe, nevertheless it was included in the model derivation since it accounts for the overall aromaticity of this class of PBR ligands.

Figures 1a–e and 2a–e show contour maps of the PLS (Partial Least Squares) coefficients and the molecular interaction fields (MIFs) for N1 and C3 probes. Due to the elimination of the repulsive (positive) interactions (see Section 5), only negative values corresponding to attractive interactions are present in our model. In the figures, blue and yellow areas correspond to negative and positive values of the parameter (PLS coefficient or MIFs, respectively. Positive areas (yellow) in the PLS coefficient plot indicate regions where a negative favourable interaction with the probe (blue MIFs) correlates with an increase in affinity (a low pK_i value), while negative PLS coefficients (blue zones) correlate with a decrease in affinity (a high pK_i value).

3.1.1. N1 probe. In the whole dataset, there are no significant variations in the capacity to accept H-bond, since the number, nature and position of these groups are not so different from one molecule to another. Nevertheless, the N1 probe groups the dataset molecules in three main classes describing them in similar ways depending on the basis of the flexible chain in the 3-position: this observation could be interpreted as a raw steric effect of the substitution in this position that influences the ability of the heteroatoms to interact with the N1 probe. Both the aliphatic (higher than methyl without distinction) and aromatic amides, forming the most populated group, are represented in Figure 1c by molecule **4b** which has the highest affinity value. The less active compounds are divided into two other groups: one, represented in Figure 1d by molecule **3b**, comprising the monosubstituted amide (**4q**), the ester (**3b**) and the dimethylamides (**4**, **4a**) and the other for the cyclized amides (**4n**, **4o** and **4p**) in Figure 1e by molecule **4p**, the least active compound of the whole dataset.

PLS coefficients contour plots highlight two blue (A, B) and one yellow region (C), which indicate interesting areas for the description of activity variations within the series. The wide blue region A corresponds to wide attractive interactions with the probe in the case of low affinity compounds **3b** and **4p** (region A in Figs. 1d and e), while this region has limited dimensions in the MIF of the active compound **4b** (Fig. 1c). The favourable interaction in this zone, which is mainly due to the presence of the heteroatom adjacent to the

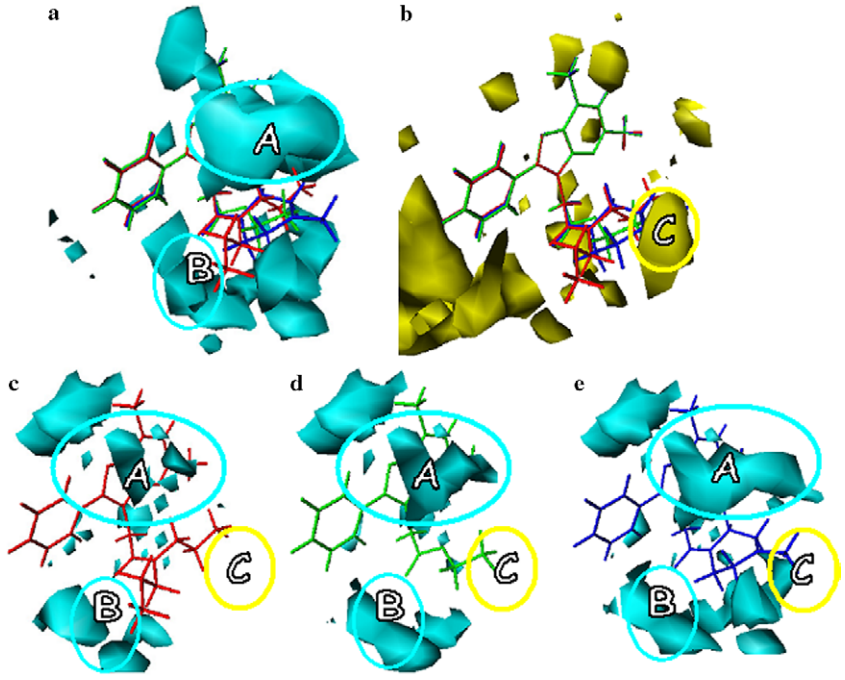


Figure 1. Probe N1 contour plots for negative (a) and positive (b) PLS coefficients together with the molecular interaction fields (MIFs) for molecules **4b** (c), **3b** (d), and **4p** (e). A–C refer to areas pointed out by the model as important for affinity.

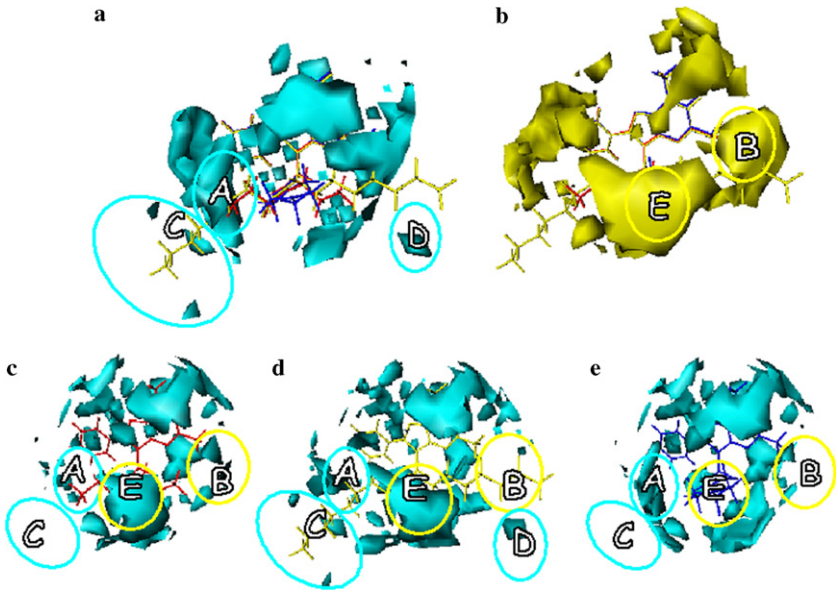


Figure 2. Probe C3 contour plots for negative (a) and positive, (b) PLS coefficients together with the molecular interaction fields (MIFs) for molecules **4b** (c), **4g** (d), and **4p** (e). A–E refer to areas pointed out by the model as important for affinity.

carbonyl group, is caused by the shielding effect of the alkyl substituents on the heteroatom: the more extended they are, the more active the compound is.

The extension of the B regions in N1-MIFs is directly related to the steric hindrance of the alkyl substituents on the acetamidic moiety and it is inversely related to the PBR affinity. The shielding effect of dialkyl substitutions greater than diethyl group (Fig. 1c) partially prevents the interaction between the carbonyl oxygen and the N1 probe, which is wider for the less active compounds bearing short dialkyl and monoalkyl chains (Fig. 1d) as well as cyclic amides (Fig. 1e).

As concerns the PLS coefficient region C, this is specifically occupied by the less active compounds that are completely unable to interact with the probe at this level (Figs. 1d and e); molecules bearing more extended aliphatic or aromatic amide chains (Fig. 1c) that are able to widen as a consequence of the steric repulsion always leave this area empty, thus making the interaction with the N1 probe possible. Such weak interactions are not shown in the figures due to the high energy threshold used to represent MIFs. The use of a lower energy cutoff would have made plot interpretation difficult. However, more insight into this aspect will be considered in the C3 probe discussion.

3.1.2. C3 probe. With respect to N1 probe, C3 is more able to discriminate molecules within the aforementioned classes. In particular, it seems to describe a sort of difference in amide chain features, supporting the hypothesis of the presence of cavities in PBR binding site with different steric tolerance, where the two alkyl substituents could be accommodated.[36,21] Such conclusions can be derived comparing both regions A and B of the PLS coefficient plots (Figs. 2a and 2b, respectively).

If molecules occupy the A region in C3-MIFs (compounds **4b** and **4g** in Figs. 2c and d, respectively), they prevent an unfavourable interaction with the probe. The model indicates a limit to the length of the chain in this position, fixed between the butylic and the pentylic chain, since higher homologues show attractive interactions in region C, which is again not favourable for the activity.

On the contrary, the occupation of area B in C3-MIFs by the molecule prevents favourable interactions with the probe (compound **4g** in Fig. 2d) in a zone of positive PLS coefficients (Fig. 2b). This could explain the progressive reduction of affinity passing from **4b** (propyl) to **4g** (octyl), since it is possible for alkyl groups higher than pentyl to interact with the C3 probe in area D, which is associated to negative values of the PLS coefficients.

Region E of Figure 2b corresponds to region C in Figure 1b. Due to the average lower energy values associated to the C3-probe, the energy cutoff used for the representation of the C3-MIFs shows significant polyhedra in correspondence of area E for molecules **4b** and **4g** (Figs. 2c and d, respectively), which were not visible in Figure 1c and are not present in the MIFs of compound **4p** (Figs. 1e and 2e).

Branching (compound **4c**) or cyclization of the alkyl chains (compound **4p**, Fig. 2e) results in a decreased PBR affinity due to the presence of the interaction area A in C3-MIFs, associated to negative PLS coefficients (Fig. 2a), together with the absence of favourable interactions in regions B and E (Fig. 2e).

The 3D-QSAR analysis provides an interpretation of the significant difference in the activity of the enantiomers **4l** (Fig. 3a) and **4m** (Fig. 3b): in fact, the more

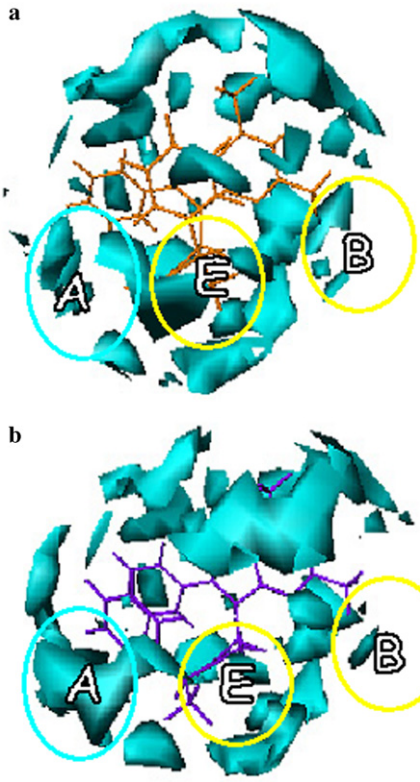


Figure 3. Probe C3 molecular interaction fields (MIFS) for molecules **4l** (a) and **4m** (b). A, B and E refer to areas pointed out by the model as important for affinity.

active R enantiomer presents extended B and E areas, associated to an increase in activity, while S enantiomer shows a wide area of interaction mainly in region A, unfavourable for affinity.

3.2. Steroidogenesis assay

Nowadays, investigation of PBR ligands activity is basically restricted to steroidogenesis assays,[37–39] even if it is well known that the PBR is involved in many cellular functions related to mitochondrial mechanisms (oxidative processes, cellular proliferation and apoptosis). Considering this observation, the inadequacy of the biological and pharmacological methods for evaluating the PBR ligands functionalities is revealed. In addition to this gap, the scanty use of PBR ligands different from the classic benzodiazepines (Ro5-4864) and PK11195 in steroidogenesis in vivo assays, which have already shown many discrepancies in acute and chronic treatments in rodents, should be kept in mind.[12] Therefore, the individuation of new PBR ligands endowed with different intrinsic activity in steroidogenesis assays in comparison to the well known Ro5-4864 and PK11195 may afford useful new opportunities to investigate the PBR functionality.The most potent compounds (**4b**, **4d** and **4j**) were examined for their ability to modulate steroid biosynthesis in C6 glioma rat cells (Fig. 4), which can represent a preliminary evaluation of the intrinsic activity of the PBR ligands. As shown in Figure 4, both PK11195 and Ro5-4864 stimulate steroid biosynthesis

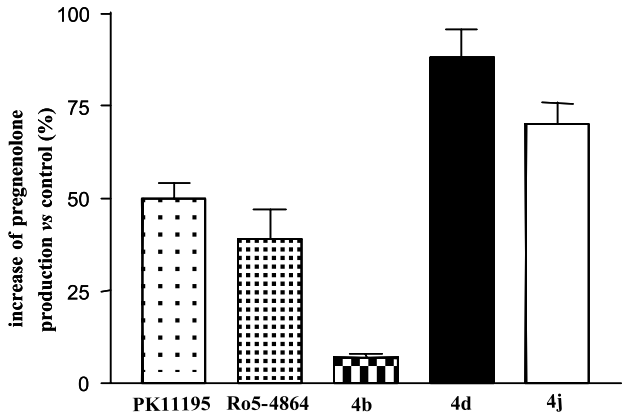


Figure 4. The effect of 2-arylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamides on the pregnenolone accumulation in C6 glioma rat cells. All the compounds were used at the same concentration (40 μM); at the end of the incubation period (2 h), the amount of pregnenolone was quantified by radio immunoassay (RIA), using an antibody obtained from ICN Biochemical Inc., CA, USA. The values are means ± SEM of at least three determinations (see Section 5).

in C6 glioma cells and they were previously shown to elicit similar effects in MA10 Leyding cells and Y-1 cells.[38] Among the tested compounds, in spite of comparable binding affinity, a different effect on steroids production can be observed.

In particular **4b**, which does not increase the level of pregnenolone, warrants further investigation in order to understand its possible antagonist profile. On the other hand, compounds **4d** and **4j** appear to function better than PK11195 and Ro5-4864 in regard to stimulation of pregnenolone production.

4. Conclusions

The series described in this paper represents a useful extension of pyrazolo[1,5-*a*]pirimidin-3-yl acetamides as PBR ligands, which offer a wide margin of modelling to identify a PBR pharmacophore. In addition to providing a pharmacophore model, we have focused our attention on the structural differences present on a molecular fragment within the series of the studied pyrazolopyrimidines, namely the acetamide chains. According to the suggestions provided by the GRID/Golpe model, work is currently in progress to further investigate these aspects through the design of new compounds bearing different substitutions on the amide nitrogen. Moreover, the GRID/Golpe model is able to explain in terms of hydrophobic interactions also the features leading to stereoselectivity at PBR binding site, thus supporting the idea of an asymmetric site with two quite different cavities.

Furthermore, the PBR ligands **4d** and **j** presented in this paper could be added to the classical benzodiazepines and isoquinolinecarboxyamides as tools in the investigation of PBR functionality. It is also noteworthy that our PBR ligand, **4b**, may present itself as an antagonist

against PK 11195 and Ro5-4864 regarding steroidogenesis.

5. Experimental

5.1. Chemistry

The structures of all compounds were supported by their ^{1}H NMR data (measured with a Varian Gemini at 200 MHz, chemical shifts expressed in δ (ppm) using DMSO-d_6 or $CDCl_3$ as solvent). The following abbreviations are used: b, broad; d, doublet; m, multiplet; q, quartet; t, triplet; and s, singlet. Melting points were determined with a Gallenkamp apparatus andwere uncorrected. Elemental analyses were performed by the laboratories of Dipartimento Farmaco-Chimico-Tecnologico of Università di Siena, Italy, with a Perkin-Elmer model 240C, elemental analyzer, and their results are within ±0.4% of theoretical values. The purity of samples was determined by means of TLC, which was performed using Machery-Nagel Duren, Alugram silica gel plates. Silica gel 60 (Merck 70–230 mesh) was used for column chromatography.

5.2. 3-Cyano-4-oxo-4-phenyl butanoic acid (1a)

To a suspension of $LiOH{\cdot}H_2O$ (10 mmol) in EtOH (50 mL) was added 3-oxo-3-phenylpropanenitrile (10 mmol) previously dissolved in EtOH (30 mL) and subsequently a solution of iodoacetic acid (12.5 mmol) and $LiOH{\cdot}H_2O$ (12.5 mmol) in 70% EtOH (50 mL) dripped into the reaction mixture; it was refluxed under magnetic stirring for 6 h. After cooling, evaporation of the solvent under reduced pressure gave a residue, that was treated with ice-water (100 mL) and the resulting mixture was washed with diethyl ether (60 mL ×3). Acidification of the aqueous phase with concentrated hydrochloric acid causes the separation of a solid product, which was isolated by filtration. Ivory crystals from H_2O; yield 32%; mp 143–144 °C; ^{1}H NMR ($CDCl_3$) δ: 3.00 (dd, 1H, CH_2), 3.29 (dd, 1H, CH_2), 4.70–4.81 (m, 1H, CH), 7.42–7.77 (m, 3H, Ph), 8.0–8.15 (m, 2H, Ph), 8.70 (br s, 1H, OH exchangeable). Anal. Calcd for $C_{11}H_9NO_3$: C, 65.02; H, 4.46; N, 6.89. Found: C, 65.13; H, 4.18; N, 6.55.

5.3. Ethyl 3-cyano-4-oxo-4-phenyl butanoate (1b)

To a solution of 3-oxo-3-phenylpropanenitrile (10 mmol) in toluene (80 mL) were added ethyl iodoacetate (15 mmol) and TlOEt (10.5 mmol) under magnetic stirring. The reaction mixture was maintained at 70 °C for 3 h. After cooling, the inorganic materials were filtered off and the evaporation of the filtrate, under reduced pressure, gave a residue that was purified by silica gel column chromatography [toluene/ethyl acetate, 8/3 (v/v), as eluent]. Orange liquid; yield 30%; ^{1}H NMR ($CDCl_3$) δ: 1.30 (t, 3H, OCH_2CH_3), 2.97 (dd, 1H, CH_2), 3.30 (dd, 1H, CH_2), 4.19 (q, 2H, OCH_2CH_3), 4.79–4.83 (m, 1H, CH), 7.43–7.77 (m, 3H, Ph), 8.01–8.19 (m, 2H, Ph). Anal. Calcd for $C_{13}H_{13}NO_3$: C, 67.52; H, 5.67; N, 6.06. Found: C, 67.47; H, 5.64; N, 6.38.

5.4. General procedure for the synthesis of compounds (2a–b)

To a solution of **1a**–**b** (10 mmol) in EtOH (50 mL), hydrazine hydrate (20 mmol, 0.97 mL) and acetic acid (1 mL) were added and the reaction mixture was refluxed for 6 h. After cooling, evaporation of the solvent, under reduced pressure, gave a residue that was purified by silica gel column chromatography [$CHCl_3$/MeOH, 10:1 v/v, as eluent].

5.5. 3-Amino-5-phenylpyrazol-4-yl acetic acid (2a)

Ivory crystals from water; obtained from compound **1a**; yield 38%; mp 200–201 °C. ^{1}H NMR (DMSO-d_6) δ: 3.38 (s, 2H, CH_2), 4.87 (br s, 2H, NH_2 exchangeable), 7.32–7.59 (m, 5H, Ph). Anal. Calcd for $C_{11}H_{11}N_3O_2$: C, 60.82; H, 5.10; N, 19.34. Found: C, 60.70; H, 4.97; N, 19.15.

5.6. Ethyl 3-amino-5-phenylpyrazol-4-yl acetate (2b)

Yellow liquid obtained from compound **1b**, yield 41%. ^{1}H NMR ($CDCl_3$) δ: 1.30 (t, 3H, OCH_2CH_3), 3.46 (s, 2H, CH_2), 4.20 (q, 2H, OCH_2CH_3), 5.80 (br s, 2H, NH_2 exchangeable), 7.42–7.54 (m, 3H, Ph), 8.09–8.12 (m, 2H, Ph). Anal. Calcd for $C_{13}H_{15}N_3O_2$: C, 63.66; H, 6.16; N, 17.13. Found: C, 63.39; H, 5.97; N, 17.15.

5.7. General procedure for the synthesis of compounds 3a and b

To a solution of **2a** and **b** (1 mmol) in EtOH (5 mL), 2,4-pentanedione (1 mmol) was added and the mixture was refluxed under stirring for 2 h. The progress of reaction was monitored by TLC. The solvent was evaporated under reduced pressure and the residue was purified by silica gel column chromatography to give the corresponding pyrazolopyrimidines **3a** and **b**.

5.8. 5,7-Dimethyl-2-phenylpyrazolo[1,5-*a*]pyrimidin-3-yl acetic acid (3a)

This compound was obtained from **2a** and purified by column chromatography ($CHCl_3$/MeOH, 20:3 v/v as eluent); yield 83%; white crystals; mp 231–232 °C. ^{1}H NMR (DMSO-d_6) δ: 2.46 (s, 3H, 5-CH_3), 2.78 (s, 3H, 7-CH_3), 3.80 (s, 2H, CH_2), 6.95 (s, 1H, H-6), 7.41–7.48 (m, 3H, Ph), 7.78–7.82 (m, 2H, Ph), 12.4 (br s, 1H, OH exchangeable). Anal. Calcd for $C_{16}H_{15}N_3O_2$: C, 68.31; H, 5.37; N, 14.94. Found: C, 68.68; H, 5.58; N, 14.99.

5.9. Ethyl 5,7-dimethyl-2-phenylpyrazolo[1,5-*a*]pyrimidin-3-yl acetate (3b)

This compound was obtained from **2b**; white crystals from EtOH; yield 25%; mp 104–105 °C. ^{1}H NMR ($CDCl_3$) δ: 1.21 (t, 3H, OCH_2CH_3), 2.62 (s, 3H, 5-CH_3), 2.81 (s, 3H, 7-CH_3), 4.01 (s, 2H, CH_2), 4.18 (q, 2H, OCH_2CH_3), 6.61 (s, 1H, H-6), 7.39–7.57 (m, 3H, Ph), 7.78–7.84 (m, 2H, Ph). Anal. Calcd for

$C_{18}H_{19}N_3O_2$: C, 69.88; H, 6.19; N, 13.58. Found: C, 70.03; H, 6.32; N, 13.65.

5.10. General procedure for the synthesis of amides (4a–q)

The 5,7-dimethyl-2-phenylpyrazolo[1,5-*a*]pyrimidin-3-yl acetic acid (**3a**) (1 mmol) and triethylamine (3.5 mmol) were dissolved in 5 mL of dry THF and cooled to −10 °C. After stirring for 30 min, to this solution was added ethyl chloroformate (1.1 mmol) and then the reaction suspension was stirred for 1 h at −10 °C before the addition of 1.1 mmol of the suitable amine. The suspension was allowed to warm to room temperature under magnetic stirring for 5 h. The reaction was quenched by introducing 5 mL of H_2O, and the resulting mixture was extracted with ether (2 × 10 mL); the ethereal extracts were washed successively with aqueous 5% HCl (5 mL) and dried over Na_2SO_4. The crude acetamide (**4a**–**q**) was obtained by removing the solvent under reduced pressure and the residue was purified by column chromatography.

5.11. *N*,*N*-Dimethyl-(5,7-dimethyl-2-phenylpyrazolo-[1,5-*a*]pyrimidin-3-yl)acetamide (4a)

This compound was obtained using *N*,*N*-dimethylamine and purified by column chromatography ($CHCl_3$/MeOH, 10:1 v/v, as eluent). Light pink crystals; yield 46%; mp 162–163°C; ^{1}H NMR ($CDCl_3$) δ (ppm): 2.58 (s, 3H, 5-CH_3), 2.78 (s, 3H, 7-CH_3), 3.00 (s, 3H, $N(CH_3)_2$), 3.18 (s, 3H, $N(CH_3)_2$), 3.95 (s, 2H, CH_2), 6.56 (s, 1H, H-6), 7.40–7.53 (m, 3H, Ph), 7.80–7.85 (m, 2H, Ph). Anal. Calcd for $C_{19}H_{22}N_4O$: C, 70.11; H, 6.54; N, 18.17. Found: C, 69.91; H, 6.29; N, 18.42.

5.12. *N*,*N*-Di-*n*-propyl-(5,7-dimethyl-2-phenylpyrazolo-[1,5-*a*]pyrimidin-3-yl)acetamide (4b)

This compound was obtained using *N*,*N*-di-*n*-propylamine and purified by column chromatography ($CHCl_3$/MeOH, 10:1 v/v, as eluent). White crystals; yield 54%; mp 129–130 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 0.82–0.98 (m, 6H, $N(CH_2CH_2CH_3)_2$), 1.43–1.78 (m, 4H, $N(CH_2CH_2CH_3)_2$), 2.58 (s, 3H, 5-CH_3), 2.78 (s, 3H, 7-CH_3), 3.28–3.47 (m, 4H, $N(CH_2CH_2CH_3)_2$), 3.98 (s, 2H, CH_2), 6.57 (s, 1H, H-6), 7.39–7.51 (m, 3H, Ph), 7.78–7.82 (m, 2H, Ph). Anal. Calcd for $C_{22}H_{28}N_4O$: C, 72.50; H, 7.74; N, 15.37. Found: C, 72.20; H, 7.50; N, 15.43.

5.13. *N*,*N*-Di-i-propyl-(5,7-dimethyl-2-phenylpyrazolo-[1,5-*a*]pyrimidin-3-yl)acetamide (4c)

This compound was obtained using *N*,*N*-di-*i*-propylamine and purified by column chromatography ($CHCl_3$/MeOH, 10:1 v/v, as eluent). White crystals; yield 22%; mp 177–178 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.17–1.36 (m, 12H, $N(CHCH_3CH_3)_2$), 2.62 (s, 3H, 5-CH_3), 2.79 (s, 3H, 7-CH_3), 3.01–3.20 (m, 2H, $N(CHCH_3CH_3)_2$), 4.01 (s, 2H, CH_2), 6.58 (s, 1H, H-6), 7.42–7.51 (m, 3H, Ph), 7.80–7.84 (m, 2H, Ph). Anal. Calcd for $C_{22}H_{28}N_4O$: C, 72.50; H, 7.74; N, 15.37. Found: C, 72.71; H, 7.83; N, 15.15.

5.14. *N*,*N*-Di-*n*-butyl-(5,7-dimethyl-2-phenylpyrazolo-[1,5-*a*]pyrimidin-3-yl)acetamide (4d)

This compound was obtained using *N*,*N*-di-*n*-butylamine and purified by column chromatography ($CHCl_3$/MeOH, 10:1 v/v, as eluent). White crystals; yield 17%; mp 95–96 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 0.82–0.98 (m, 6H, $N[(CH_2)_3CH_3]_2$), 1.21–1.38 (m, 4H, $N[(CH_2)_3CH_3]_2$), 1.47–1.63 (m, 4H, $N[(CH_2)_3CH_3]_2$), 2.59 (s, 3H, 5-CH_3), 2.79 (s, 3H, 7-CH_3), 3.29–3.44 (m, 4H, $N[(CH_2)_3CH_3]_2$), 3.97 (s, 2H, CH_2), 6.58 (s, 1H, 6-H), 7.39–7.44 (m, 3H, Ph), 7.79–7.86 (m, 2H, Ph). Anal. Calcd for $C_{24}H_{32}N_4O$: C, 73.43; H, 8.22; N, 14.27. Found: C, 73.13; H, 8.02; N, 14.01.

5.15. *N*,*N*-Di-*n*-pentyl-(5,7-dimethyl-2-phenylpyrazolo-[1,5-*a*]pyrimidin-3-yl)acetamide (4e)

This compound was obtained using *N*,*N*-di-*n*-pentylamine and purified by column chromatography ($CHCl_3$/MeOH, 20:1 v/v, as eluent). White crystals; yield 34%; mp 92–93 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 0.80–0.98 (m, 6H, $N[(CH_2)_4CH_3]_2$), 1.17–1.38 (m, 6H, $N[(CH_2)_4CH_3]_2$), 1.53–1.64 (m, 6H, $N[(CH_2)_4CH_3]_2$), 2.58 (s, 3H, 5-CH_3), 2.78 (s, 3H, 7-CH_3), 3.23–3.42 (m, 4H, $N[(CH_2)_4CH_3]_2$), 3.94 (s, 2H, CH_2), 6.58 (s, 1H, 6-H), 7.40–7.52 (m, 3H, Ph), 7.78–7.87 (m, 2H, Ph). Anal. Calcd for $C_{26}H_{36}N_4O$: C, 74.25; H, 8.63; N, 13.32. Found: C, 74.15; H, 8.52; N, 13.11.

5.16. *N*,*N*-Di-*n*-hexyl-(5,7-dimethyl-2-phenylpyrazolo-[1,5-*a*]pyrimidin-3-yl)acetamide (4f)

This compound was obtained using *N*,*N*-di-*n*-hexylamine and purified by column chromatography ($CHCl_3$/MeOH, 20:1 v/v, as eluent). Uncoloured liquid; yield 34%. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.82–0.97 (m, 6H, $N[(CH_2)_5CH_3]_2$), 1.20–1.28 (m, 10H, $N[(CH_2)_5CH_3]_2$), 2.58 (s, 3H, 5-CH_3), 2.78 (s, 3H, 7-CH_3), 3.26–3.42 (m, 4H, $N[(CH_2)_5CH_3]_2$), 3.92 (s, 2H, CH_2), 6.58 (s, 1H, 6-H), 7.38–7.48 (m, 3H, Ph), 7.79–7.89 (m, 2H, Ph). Anal. Calcd for $C_{28}H_{40}N_4O$: C, 74.96; H,8.99; N, 12.49. Found: C, 74.75; H, 8.62; N, 12.11.

5.17. *N*,*N*-Di-*n*-octyl-(5,7-dimethyl-2-phenylpyrazolo-[1,5-*a*]pyrimidin-3-yl)acetamide (4g)

This compound was obtained using *N*,*N*-di-*n*-octylamine and purified by column chromatography ($CHCl_3$/MeOH, 10:0.3 v/v, as eluent). White waxed solid; yield 41%. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.85–0.90 (m, 6H, $N[(CH_2)_7CH_3]_2$), 1.14–1.38 (m, 20H, $N[(CH_2)_5CH_3]_2$), 1.46–1.61 (m, 4H, $N[(CH_2)_5CH_3]_2$), 2.56 (s, 3H, 5-CH_3), 2.75 (s, 3H, 7-CH_3), 3.27–3.40 (m, 4H, $N[(CH_2)_5CH_3]_2$), 3.94 (s, 2H, CH_2), 6.52 (s, 1H, 6-H), 7.37–7.82 (m, 5H, Ph). Anal. Calcd for $C_{32}H_{48}N_4O$: C, 76.15; H, 9.59; N, 11.10. Found: C, 76.45; H, 9.62; N, 11.37.

5.18. *N*,*N*-Dibenzyl-(5,7-dimethyl-2-phenylpyrazolo-[1,5-*a*]pyrimidin-3-yl)acetamide (4h)

This compound was obtained using *N*,*N*-dibenzyl and purified by column chromatography (EtOAc as eluent).

White waxed solid; yield 48%. ^{1}H NMR ($CDCl_3$) δ (ppm): 2.55 (s, 3H, 5-CH_3), 2.72 (s, 3H, 7-CH_3), 4.07 (s, 2H, CH_2), 4.61 (s, 2H, CH_2Ph), 4.64 (s, 2H, CH_2Ph), 6.51 (s, 1H, 6-H), 7.12–7.45 (m, 13H, Ph), 7.78–7.85 (m, 2H, Ph). Anal. Calcd for $C_{30}H_{28}N_4O$: C, 78.23; H, 6.13; N, 12.16. Found: C, 78.36; H, 6.25; N, 11.98.

5.19. *N*-Ethyl-*N*-i-propyl-(5,7-dimethyl-2-phenylpyrazolo-[1,5-*a*]pyrimidin-3-yl)acetamide (4i)

This compound was obtained using *N*-ethyl-*N*-*i*-propylamine and purified by column chromatography ($CHCl_3$/MeOH, 20:1 v/v, as eluent). Light yellow crystals; yield 35%; mp 130–131 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.10–1.36 (m, 9H: 3H, N(CH_2CH_3); 6H, N($CH(CH_3)_2$), 2.58 (s, 3H, 5-CH_3), 2.78 (s, 3H, 7-CH_3), 3.20–3.56 (m, 2H, N(CH_2CH_3)), 3.90–4.00 (m, 2H, CH_2), 4.36–4.80 (m, 1H, N($CH(CH_3)_2$), 6.57 (s, 1H, 6-H), 7.38–7.50 (m, 3H, Ph), 7.78–7.85 (m, 2H, Ph). Anal. Calcd for $C_{21}H_{26}N_4O$: C, 71.97; H, 7.48; N, 15.99. Found: C, 72.06; H, 7.27; N, 15.90.

5.20. *N*-Ethyl-*N*-phenyl-(5,7-dimethyl-2-phenylpyrazolo-[1,5-*a*]pyrimidin-3-yl)acetamide (4j)

This compound was obtained using *N*-ethyl-*N*-phenylamine and purified by column chromatography ($CHCl_3$/MeOH, 20:1 v/v, as eluent). White crystals; yield 24%; mp 153–154 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.10 (t, 3H, N(CH_2CH_3)), 2.58 (s, 3H, 5-CH_3), 2.68 (s, 3H, 7-CH_3), 3.65–3.81 (m, 4H: 2H, CH_2; 2H, N(CH_2CH_3)), 6.56 (s, 1H, ,6-H), 7.30–7.56 (m, 8H: 3H, Ph; 5H, N-Ph), 7.70–7.80 (m, 2H, Ph). Anal. Calcd for $C_{24}H_{24}N_4O$: C, 74.97; H, 6.29; N, 14.57. Found: C, 75.16; H, 6.27; N, 14.87.

5.21. *N*-Benzyl-*N*-ethyl-(5,7-dimethyl-2-phenylpyrazolo-[1,5-*a*]pyrimidin-3-yl)acetamide (4k)

This compound was obtained using *N*-benzyl-*N*-ethylamine and purified by column chromatography ($CHCl_3$/MeOH, 20:1 v/v, as eluent). White crystals; yield 20%; mp 141–142 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.10–1.30 (m, 3H, N(CH_2CH_3)), 2.84–2.89 (m, 6H: 3H, 5-CH_3; 3H, 7-CH_3), 3.40–3.60 (m, 2H, N(CH_2CH_3)), 4.31 (d, 2H, CH_2), 4.73 (d, 2H, NCH_2Ph), 6.60–6.75 (m, 1H, 6-H), 7.20–7.27 (m; 5H, N CH_2Ph), 7.40–7.58 (m, 3H, Ph), 7.70–7.83 (m, 2H, Ph). Anal. Calcd for $C_{25}H_{26}N_4O$: C, 75.35; H, 6.58; N, 14.06. Found: C, 75.24; H, 6.98; N, 13.89.

5.22. (*R*)-(+)-*N*,α-Dimethylbenzyl-(5,7-dimethyl-2-phenyl-pyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (4l)

This compound was obtained using (R)-(+)-N,α-Dimethylbenzylamine and purified by column chromatography ($CHCl_3$/MeOH, 20:1 v/v, as eluent). White crystals; yield 56%; mp 104–106 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.50 (d, 3H, CH_3), 2.6–2.84 (m, 9H: 5-CH_3, 7-CH_3, N-CH_3), 4.01–4.22 (m, 2H, CH_2), 6.10 (q, 1H, CH), 6.62 (s, 1H, 6-H), 7.22–7.58 (m, 8H: 5H, Ph; 3H, Ph), 7.78–7.91 (m, 2H, Ph). Anal. Calcd for $C_{25}H_{26}N_4O$: C, 75.35; H, 6.58; N, 14.06. Found: C, 75.50; H, 6.88; N, 13.95.

5.23. (*S*)-(−)-*N*,α-Dimethylbenzyl-(5,7-dimethyl-2-phenyl-pyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (4m)

This compound was obtained using (S)-(-)-N,α-Dimethylbenzylamine and purified by column chromatography ($CHCl_3$/MeOH, 20:1 v/v, as eluent). White crystals; yield 51%; mp 104—106 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.53 (d, 3H, CH_3), 2.70–2.90 (m, 9H: 5-CH_3, 7-CH_3, N-CH_3), 4.01–4.22 (m, 2H, CH_2), 6.10 (q, 1H, CH), 6.63 (s, 1H, 6-H), 7.22–7.38 (m, 5H, Ph), 7.41–7.58 (m, 3H, Ph), 7.78–7.91 (m, 2H, Ph). Anal. Calcd for $C_{25}H_{26}N_4O$: C, 75.35; H, 6.58; N, 14.06. Found: C, 75.67; H, 6.43; N, 14.26.

5.24. 5,7-Dimethyl-2-phenyl-3-[(pyrrolidinocarbonyl)-methyl]pyrazolo[1,5-*a*]pyrimidine (4n)

This compound was obtained using pyrrolidine and purified by column chromatography ($CHCl_3$/MeOH, 20:1 v/v, as eluent). White crystals; yield 85%; mp 191–192 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.81–2.03 (m, 4H, N$(CH_2)_4$), 2.58 (s, 3H, 5-CH_3), 2.78 (s, 3H, 7-CH_3), 3.43–3.70 (m, 4H, N$(CH_2)_4$), 43.86 (s, 2H, CH_2), 6.56 (s, 1H, 6-H), 7.39–7.50 (m, 3H, Ph), 7.83–7.91 (m, 2H, Ph). Anal. Calcd for $C_{20}H_{22}N_4O$: C, 71.83; H, 6.63; N, 16.75. Found: C, 71.98; H, 6.57; N, 16.60.

5.25. 5,7-Dimethyl-2-phenyl-3-[(piperidinocarbonyl)methyl]pyrazolo[1,5-*a*]pyrimidine (4o)

This compound was obtained using piperidine and purified by column chromatography ($CHCl_3$/MeOH, 20:1 v/v, as eluent). Ivory crystals; yield 33%; mp 177–178 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.44–1.72 (m, 6H, N$(CH_2)_5$), 2.59 (s, 3H, 5-CH_3), 2.79 (s, 3H, 7-CH_3), 3.50–3.63 (m, 4H, N$(CH_2)_5$), 3.98 (s, 2H, CH_2), 6.58 (s, 1H, 6-H), 7.37–7.53 (m, 3H, Ph), 7.79–7.90 (m, 2H, Ph). Anal. Calcd for $C_{21}H_{24}N_4O$: C, 72.39; H, 6.94; N, 16.08. Found: C, 72.67; H, 7.10; N, 15.97.

5.26. 5,7-Dimethyl-3-[(4-methylpiperazinocarbonyl)methyl]-2-phenyl-pyrazolo[1,5-*a*]pyrimidine (4p)

This compound was obtained using *N*-methylpiperazine and purified by column chromatography ($CHCl_3$/MeOH, 10:1 v/v, as eluent). White crystals; yield 13%; mp 156–157 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 2.36 (s, 3H, NCH_3), 2.38–2.44 (m, 4H, NN$(CH_2)_4$), 2.58 (s, 3H, 5-CH_3), 2.78 (s, 3H, 7-CH_3), 3.07 (m, 4H, NN$(CH_2)_4$), 3.96 (s, 2H, CH_2), 6.58 (s, 1H, H-6), 7.42–7.48 (m, 3H, Ph), 7.80–7.84 (m, 2H, Ph). Anal. Calcd for $C_{21}H_{25}N_5O$: C, 69.40; H, 6.93; N, 19.27. Found: C, 69.15; H, 7.05; N, 19.59.

5.27. *N*-Propyl-(5,7-dimethyl-2-phenylpyrazolo[1,5-*a*]-pyrimidin-3-yl)acetamide (4q)

This compound was obtained using propylamine and purified by column chromatography ($CHCl_3$/MeOH,

10:1 v/v, as eluent). Ivory crystals; yield 57%; mp 188–189 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 0.82 (t, 3H, N-$CH_2CH_2CH_3$), 1.26–1.34 (m, 2H, N-$CH_2CH_2CH_3$), 2.60 (s, 3H, 5-CH_3), 2.81 (s, 3H, 7-CH_3), 3.16–3.24 (m, 2H, N-$CH_2CH_2CH_3$), 3.83 (s, 2H, CH_2), 6.61 (s, 1H, H-6), 6.81 (br s, 1H, NH exchangeable), 7.42–7.58 (m, 3H, Ph), 7.86–8.01 (m, 2H, Ph). Anal. Calcd for $C_{19}H_{22}N_4O$: C, 70.78; H, 6.88; N, 17.38. Found: C, 70.63; H, 6.59; N, 17.64.

5.28. *N*,*N*-Diethyl-(5,7-dimethyl-2-phenylpyrazolo[1,5-*a*]-pyrimidin-3-yl)tioacetamide (5)

To a solution of *N*,*N*-diethyl-(2-phenyl-5,7-dimethyl-pyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (**3i**)[18] (1 mmol) in toluene (25 mL), Lawesson's reagent (2 mmol) was added and the reaction mixture was refluxed under magnetic stirring for 2 h. The resulting solution was treated with 5% $NaHCO_3$ and the organic phase was washed with water and then evaporated under reduced pressure; the residue was purified by silica gel column chromatography ($CHCl_3$/MeOH, 20:1 v/v, as eluent). White crystals; yield 30%; mp 136–137 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.26–1.32 (m, 6H, N(CH_2CH_3)$_2$), 2.55 (s, 3H, 5-CH_3), 2.77 (s, 3H, 7-CH_3), 3.82 (q, 2H, N(CH_2CH_3)$_2$), 4.07 (q, 2H, N(CH_2CH_3)$_2$), 4.28 (s, 2H, CH_2), 6.54 (s, 1H, H-6), 7.42–7.46 (m, 3H, Ph), 7.85–7.88 (m, 2H, Ph). Anal. Calcd for $C_{20}H_{24}N_4S$: C, 68.15; H, 6.86; N, 15.89. Found: C, 68.01; H, 7.09; N, 15.72.

5.29. 5,7-Dimethyl 2-phenylpyrazolo[1,5-*a*]pyrimidin-3-carboxamide (6)

The 5,7-dimethyl-2-phenylpyrazolo[1,5-*a*]pyrimidin-3-carbonitrile[25] (2.62 mmol) was dissolved in H_2SO_4 (5 mL) and the resulting solution was maintained at room temperature for 1 h, under magnetic stirring. To the reaction mixture was added ice water and compound **6** was isolated by filtration and copiously washed with water. The crude amide was purified by column chromatography ($CHCl_3$/MeOH, 10:1 v/v, as eluent). White crystals; yield 89%; mp 252–253 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 2.64 (s, 3H, 5-CH_3), 2.82 (s, 3H, 7-CH_3), 5.57 (s, 1H, NH_2), 6.77 (s, 1H, 6-H), 7.42–7.57 (m, 3H, Ph), 7.92–8.17 (m, 2H, Ph), 8.57 (s, 1H, NH_2). Anal. Calcd for $C_{17}H_{22}N_4O$: C, 68.43; H, 7.43; N, 18.78. Found: C, 68.10; H, 7.19; N, 18.72.

5.30. 5,7-Dimethyl-2-phenylpyrazolo[1,5-*a*]pyrimidin-3-carboxylic acid (7)

A saturated solution of $NaNO_2$ was added slowly to a solution of compound **6** (1.3 mmol) in H_2SO_4 (5 mL) kept at −5 °C. The resulting suspension was stirred for 30 min and then extracted with ether (2× 10 mL); the ethereal extracts were dried over Na_2SO_4. The crude acid **7** was obtained by removing the solvent under reduced pressure and purified by column chromatography ($CHCl_3$/MeOH, 20:3 v/v, as eluent). Ivory crystals; yield 27%; mp: 188 °C; ^{1}H NMR (DMSO-d_6) δ (ppm): 2.59 (s, 3H, 5-CH_3), 2.71 (s, 3H, 7-CH_3), 7.19 (s, 1H, 6-H), 7.60–7.82 (m, 3H, Ph), 7.99–8.38 (m, 2H, Ph), 12.29 (s, 1H, COOH). Anal. Calcd for $C_{17}H_{21}N_3O_2$: C, 68.20; H, 7.07; N, 14.04. Found: C, 68.10; H, 7.18; N, 13.92.

5.31. *N*,*N*-Diethyl-5,7-dimethyl-2-phenylpyrazolo[1,5-*a*]-pyrimidin-3-carboxamide (8)

Compound **7** (1 mmol) and triethylamine (3.5 mmol) were dissolved in 5 mL of dry THF and cooled to −10 °C. After stirring for 30 min, to this solution was added ethyl chloroformate (1.1 mmol) and then the reaction suspension was stirred for 1 h at −10 °C before the addition of 1.1 mmol of the suitable amine. The suspension was allowed to warm to room temperature under magnetic stirring for 5 h. The reaction was quenched by introducing 5 mL of H_2O, and the resulting mixture was extracted with ether (2 × 10 mL); the ethereal extracts were washed successively with aqueous 5% HCl (5 mL) and dried over Na_2SO_4. The crude amide **8** was obtained by removing the solvent under reduced pressure and purified by column chromatography ($CHCl_3$/MeOH, 20:1 v/v, as eluent). Yellow crystals; yield 35%; mp 180–181 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.02 (t, 3H, N(CH_2CH_3)$_2$), 1.37 (t, 3H N(CH_2CH_3)$_2$), 2.60 (s, 3H, 5-CH_3), 2.81 (s, 3H, 7-CH_3), 3.26 (d, 2H, N(CH_2CH_3)$_2$) 3.76 (d, 2H, N(CH_2CH_3)$_2$), 6.68 (s, 1H, 6-H), 8.10–8.20 (m, 3H, Ph), 8.23–8.35 (m, 2H, Ph). Anal. Calcd for $C_{21}H_{30}N_4O$: C, 71.15; H, 8.53; N, 15.80. Found: C, 71.10; H, 8.19; N, 15.72.

5.32. 3D QSAR analysis

Calculations were performed on a AMD Athlon XP 1800+ personal computer. All molecular modelling and QSAR studies were performed on a SGI O2 R10000 workstation. The structures of all studied compounds were generated using fragment libraries and/or the Builder module of the InsightII2000 package.[40] The energies of the molecules were minimized with conjugate gradient procedure using cvff forcefield (FF) and Discover module of InsightII 2000. The biological activity of the compounds was homogeneously expressed as binding affinity (K_i) and transformed to the ($-\log K_i$) values for using in the QSAR analysis. The conformational search was performed using a simulated annealing (SA) procedure which was started using cvff FF, distant dependent dielectric constant and a convergence criterion of 0.001 kcalmol^{-1} Å^{-1}. The molecular dynamic (MD) simulation took 1000 fs to reach 900 K. The system was then kept at 900 K for 2000 fs and subsequently cooled down at 400 K, at regular intervals, by decreasing the simulation temperature. Molecules were then allowed to minimize in order to reach the final minimum conformation. The described cycle was repeated 100 times so that 100 energy-minimized conformers for each PBR inhibitor were obtained. A cluster analysis was performed using an 'in house' developed routine written in Visual Basic programming language version 6.0 (Microsoft Corporation) that groups molecules on the basis of the root mean square (rms) value of the distance between atoms. The alignment of PBR inhibitors was accomplished by superposing the common bicyclic core. Single conformers of each molecule were obtained so that all of them present the same orientation of the ace-

tamidic group and most of them correspond to lower energy state. Molecules of the training set were selected according to statistical molecular design principles carried out in the space of the principal component scores of the Volsurf[41] descriptors so that the resulting 27 selected compounds provided actually an adequate coverage of the whole structural space. Molecular interaction fields (MIFs) were computed with GRID programme[36] using C3, DRY and N1 probes and the following parameters: 1 Å grid spacing, $26 \times 30 \times 20$ grid box dimension. PCA (Principal Component Analysis) and PLS (Partial Least Squares) algorithm were used as implemented in Golpe programme[23] on the matrix derived from the unfolding of the probe-ligand interactions into vectors (one for each ligand considered). This matrix was submitted to Golpe advanced pretreatment including max cutoff = 0 kcal, zeroing = 0.01 kcal, Min sd = 0.02 Å, N-level = 2, 3 and 4. The resulting 10,016 variables were normalized according to the BUW procedure. The smart region definition (number of seed = 1757, critical distance cutoff = 1.0 Å, collapsing cutoff = 2.0 Å) in combination with the fractional factorial design was used in the variable selection. PCA based on C3 and N1 probes was carried out: no outliers were found. The optimal dimensionality of the PLS model was determined according to leave-one-out (LOO) and random groups cross-validation procedure.

5.33. Binding to rat kidney mitochondrial membranes

For binding studies, mitochondria were prepared as previously described[31,19] with minor modification as described below, from kidneys of Male Wistar rats killed by cervical dislocation. Kidneys were homogenized in 20 volumes of ice-cold 50 mM Tris/HCl, pH 7.4, 0.32 M sucrose and 1 mM EDTA (buffer A), containing protease inhibitors (160 μg/mL benzamidine, 200 μg/mL bacitracine and 20 μg/mL soybean trypsin inhibitor) with a Teflon pestle in a glass homogenizer and centrifuged at 600*g* for 10 min at 4 °C. The resulting supernatant was centrifuged at 10,000*g* for 10 min at 4 °C. The pellet was then resuspended in 20 volumes of ice-cold buffer A, and centrifuged again at 10,000*g* for 10 min at 4 °C. The crude mitochondrial pellet was frozen at −20 °C until the time of assay or incubated with either 0.6 nM [^{3}H]PK11195 or 1 nM [^{3}H]Ro 5-4864 in 50 mM Tris/HCl, pH 7.4 (buffer B), with a range of concentrations of the tested compounds (0.1 nM to 10 μM) in a total volume of 0.5 mL for 90 min at 4 °C. The incubation was terminated by dilution to 5 mL with ice-cold buffer B, followed immediately by rapid filtration through glass fiber Whatman GF/C filters. The filters were then washed (2× 5 mL) with buffer B and the amount of radioactivity retained on the filters was determined by Packard 1600 TR liquid scintillation counter at 66% efficiency. Non-specific binding was estimated in each case in the presence, respectively, of unlabeled 1 μM PK 11195 or Ro5-4864. For the active compounds, the IC_{50} values were determined and K_i values were derived according to the equation of Cheng and Prusoff.[42] Protein concentration was estimated by the method of Lowry et al.[43] with bovine serum as standard.

5.34. [^{3}H]Ro15-1788 binding to rat cerebral cortex membranes

Rat cerebral cortex membranes were prepared as previously described.[44] After differential centrifugation, the obtained crude membrane fraction was subjected to washing procedures to remove endogenous GABA.[45] The washed membranes were incubated with 0.2 nM [^{3}H]Ro 15-1788 for 90 min at 0 °C in 500 μL of buffer, 50 mM Tris–citrate buffer, pH 7.4, as previously described.[46]

5.35. Cell culture

Rat glioma C6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. Cultures were maintained in a humidified atmosphere of 5% CO_2/95% air at 37°C.

5.36. Steroid biosynthesis

C6 cells were seeded in 24-well plates at a density of $\sim 1 \times 10^6$ cells/well in a final volume of 1 mL. Prior to measurement of pregnenolone production, the cells were washed three times with a simple salts medium consisting of 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM glucose, 10 mM HEPES/NaOH, pH 7.4, plus 0.1% BSA. During experiments, cells were incubated with this simple salts medium in air incubator at 37 °C. In order to measure pregnenolone secreted into the medium, its further metabolism was blocked by the addition of trilostane (25 μM) and SU 10603 (10 μM) (inhibitors of 3β-hydroxysteroid dehydrogenase and 17α-hydroxylase, respectively) to the simple salts medium, as previously described.[19] The addition of the novel compounds and of PK 11195, Ro5-4864, or clonazepam to the C6 cells was made by the complete change of the simple salts medium to medium containing the appropriate concentration (40 μM) of compound. The final concentration of ethanol was constant for all the wells within each experiment and did not exceed 0.5% (v/v), a concentration which on its own had no effect on steroid production. At the end of the incubation period (2 h), the cell medium was retained and centrifuged at 1500 g for 10 min. The amount of pregnenolone secreted into the medium was quantified by radio immunoassay (RIA), using an antibody obtained from ICN Biochemical Inc., CA, U.S.A., under the conditions recommended by the supplier. Cell protein concentration was measured according to the method of Lowry et al.[43]

Acknowledgment

This research was supported by funds of the Italian Ministry of University and Scientific Research (MURST).

References and notes

1. McEnery, M. W.; Snowman, A. M.; Trifiletti, R. R.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3170.

2. Casellas, P.; Galiegue, S.; Basile, A. S. *Neurochem. Int.* **2002**, *40*, 475.
3. Krueger, K. E. *Biochim. Biophys. Acta* **1995**, *1241*, 453.
4. Gavish, M.; Bachman, I.; Shoukrun, R.; Katz, Y.; Veenman, L.; Weisinger, G.; Weizman, A. *Pharmacol. Rev.* **1999**, *51*, 629.
5. O'Beirne, G. B.; Williams, D. C. *Eur. J. Biochem.* **1988**, *175*, 413.
6. Galiegue, S.; Tinel, N.; Casellas, P. *Curr. Med. Chem.* **2003**, *10*, 1563.
7. Veenman, L.; Leschiner, S.; Spanier, I.; Weisinger, G.; Weizman, A.; Gavish, M. *J. Neurochem.* **2002**, *80*, 917.
8. Avital, A.; Richter-Levin, G.; Leschiner, S.; Spanier, I.; Veenman, L.; Weizman, A.; Gavish, M. *Neuropsychopharmacology* **2001**, *25*, 669.
9. Kelly-Hershkovitz, E.; Weizman, R.; Spanier, I.; Leschiner, S.; Lahav, M.; Weisinger, G.; Gavish, M. *J. Biol. Chem.* **1998**, *273*, 5478.
10. Lang, S. *Curr. Med. Chem.* **2002**, *9*, 1411.
11. Hauet, T.; Liu, J.; Li, H.; Gazouli, M.; Culty, M.; Papadopoulos, V. *Endocr. Res.* **2002**, *28*, 395.
12. Lacapere, J. J.; Papadopoulos, V. *Steroids* **2003**, *68*, 569.
13. Liu, J.; Li, H.; Papadopoulos, V. *J. Steroid. Biochem. Mol. Biol* **2003**, *85*, 275.
14. Gazouli, M.; Han, Z.; Papadopoulos, V. *J. Pharmacol. Exp. Ther.* **2002**, *303*, 627.
15. Farges, R.; Joseph-Liauzun, E.; Shire, D.; Caput, D.; Le Fur, G.; Ferrara, P. *Mol. Pharmacol.* **1994**, *46*, 1160.
16. Yeliseev, A. A.; Kaplan, S. *J. Biol. Chem.* **1999**, *274*, 21234.
17. Papadopoulos, V.; Amri, H.; Boujrad, N.; Cascio, C.; Culty, M.; Garnier, M.; Hardwick, M.; Li, H.; Vidic, B.; Brown, A. S.; Reversa, J. L.; Bernassau, J. M.; Drieu, K. *Steroids* **1998**, *62*, 21.
18. Cinone, N.; Höltje, H.-D.; Carotti, A. *J. Comput. Aided Drug Des.* **2000**, *14*, 753.
19. Campiani, G.; Nacci, V.; Fiorini, I.; De Filippis, M. P.; Garofalo, A.; Ciani, S. M.; Greco, G.; Novellino, E.; Williams, D. C.; Zisterer, D. M.; Woods, M. J.; Mihai, C.; Manzoni, C.; Mennini, T. *J. Med. Chem.* **1996**, 3435.
20. Cappelli, A.; Anzini, M.; Vomero, S.; De Benedetti, P. G.; Menziani, M. C.; Giorgi, G.; Manzoni, C. *J. Med. Chem.* **1997**, *40*, 2910.
21. Anzini, M.; Cappelli, A.; Vomero, S.; Seeber, M.; Menziani, M. C.; Langer, T.; Hagen, B.; Manzoni, C.; Bourguignon, J.-J. *J. Med. Chem.* **2001**, *44*, 1134.
22. Selleri, S.; Bruni, F.; Costagli, C.; Costanzo, A.; Guerrini, G.; Ciciani, G.; Costa, B.; Martini, C. *Bioorg. Med. Chem.* **2001**, *9*, 2661.
23. GOLPE 4.5. Multivariate Infometric Analysis Srl. (http://www.miasrl.com).
24. Taylor, E. C.; Hawks, G. H.; McKillop, A. *J. Am. Chem. Soc.* **1968**, *90*, 2421.
25. Mansour, T. S. *Tetrahedron Lett.* **1988**, *29*, 3437.
26. Ansell, M. F. In *Preparation of Acyl Halides*; Interscience: London, Engl., 1972, pp 35–68.
27. Jang, D. O.; Park, D. J.; Kim, J. *Tetrahedron Lett.* **1999**, *40*, 5323.
28. Frøyen, P. *Synth. Commun.* **1995**, *25*, 959.
29. Ghozlan Said, A. S. *Pharmazie* **1991**, *46*, 352.
30. Romeo, E.; Auta, J.; Kozikowski, A. P.; Ma, D.; Papadopoulos, V.; Puia, G.; Costa, E.; Guidotti, A. *J. Pharmacol. Exp. Ther.* **1992**, *262*, 971.
31. Trapani, G.; Franco, M.; Ricciardi, L.; Latrofa, A.; Genchi, G.; Sanna, E.; Tuberi, F.; Cagetti, E.; Biggio, G.; Liso, G. *J. Med. Chem.* **1997**, *40*, 3109.
32. Dubroeucq, M. C.; Benavides, J.; Doble, A.; Guillox, F.; Allam, D.; Vaucher, N.; Bertran, P.; Gueremy, C.; Renault, C.; Uzan, A. *Eur. J. Pharmacol.* **1986**, *128*, 269.
33. Newman, A. H.; Lueddens, H. W.; Skolnick, P.; Rice, K. C. *J. Med. Chem.* **1987**, *30*, 1901.
34. Shah, F.; Hume, S. P.; Pike, V. W.; Ashworth, S.; McDermott, J. *Nucl. Med. Biol.* **1994**, *21*, 573.
35. Selleri, S.; Bruni, F.; Costagli, C.; Costanzo, A.; Guerrini, G.; Ciciani, G.; Gratteri, P.; Besnard, F.; Costa, B.; Martini, C.; Fohlin, J.; De Siena, G.; Malmberg Aiello, P.; *J. Med. Chem.*, submitted for publication.
36. (a) Goodford, P. J. *J. Med. Chem.* **1985**, *28*, 849; (b) GRIDv.20 Molecular Discovery Ltd (http://www.moldiscovery.com).
37. Trapani, G.; Franco, M.; Latrofa, A.; Ricciardi, L.; Carotti, A.; Serra, M.; Sanna, E.; Biggio, G.; Liso, G. *J. Med. Chem.* **1999**, *42*, 3934.
38. Campiani, G.; Ramunno, A.; Fiorini, I.; Nacci, V.; Morelli, E.; Novellino, E.; Goegan, M.; Mennini, T.; Sullivan, S.; Zisterer, D. M.; Williams, C. D. *J. Med. Chem.* **2002**, *45*, 4276.
39. Primofiore, G.; Da Settimo, F.; Taliani, S.; Simorini, F.; Patrizi, M. P.; Novellino, E.; Greco, G.; Abignente, E.; Costa, B.; Chelli, B.; Martini, C. *J. Med. Chem.* **2002**, *45*, 4276.
40. InsightII 2000, Accelrys Inc. (www.accelrys.com).
41. (a) Volsurf v.3.0.11. (http://www.moldiscovery.com); (b) Cruciani, G.; Crivori, P.; Carrupt, P.-A.; Testa, B. *J. Mol. Struct. Theochem.* **2000**, *503*, 17.
42. Cheng, Y.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099.
43. Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
44. Squires, R. F.; Braestrup, C. *Nature* **1997**, *266*, 732.
45. Karobath, M.; Sperk, G. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 1004.
46. Costanzo, A.; Guerrini, G.; Ciciani, G.; Bruni, F.; Selleri, S.; Costa, B.; Martini, C.; Lucacchini, A.; Aiello, P. M.; Ipponi, A. *J. Med. Chem.* **1999**, *42*, 2218.